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Tmub1 negatively regulates liver regeneration via inhibiting STAT3 phosphorylation

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ABSTRACT

Tmub1 (transmembrane and ubiquitin-like domain-containing 1) plays negative roles in rat hepatocyte proliferation, but its underlying molecular mechanisms in liver regeneration regulation have yet to be revealed. Here, we show that in vivo transfection of Tmub1 overexpression vectors impaired mouse liver regeneration after partial hepatectomy (PHx). Loss- and gain-of-function analyses in human hepatocyte Lo2 cells indicated that Tmub1 inhibits the phosphorylation of STAT3 and the activation of STAT3 signaling. Furthermore, the inhibitory effect of Tmub1 overexpression on hepatocyte proliferation can be reversed by the STAT3 activator OSM, while the promotive effect of Tmub1 knockdown can be abolished by the STAT3 inhibitor stattic. Coimmunoprecipitation assays revealed interaction between Tmub1 and STAT3. Finally, we present data from chromatin immunoprecipitation and luciferase reporter gene assays and report that STAT3 binds to and activates the promoter of Tmub1, suggesting a putative negative feedback loop between Tmub1 and STAT3 signaling. Taken together, the results of our study suggest that Tmub1 is an important negative regulator of hepatocyte proliferation in liver regeneration through STAT3 signaling. These findings provide a potential strategy for the management of liver regeneration.

1. Introduction

The liver has a remarkable capacity to regenerate. Upon liver injury or partial hepatectomy, normal hepatocytes re-enter the cell cycle and proliferate to restore the original liver volume, mass and function [1]. Despite the potent regenerative capacity of the liver, nearly one million deaths per year are associated with chronic liver failure, indicating that, in these cases, liver regeneration was not sufficient to compensate for the loss of hepatocytes or to restore liver function [2]. Additionally, although partial hepatectomy (PHx) is a curative treatment for hepatic tumors and other liver diseases, large-volume liver resection may cause liver failure and ultimately result in poor outcomes [3,4]. Therefore, the use of novel therapeutic targets that effectively stimulate liver regeneration would be an ideal approach to prevent and treat liver failure.

Liver regeneration is a highly orchestrated process that is regulated by multiple inflammatory cytokines, growth factors and hormones. In the initiation phase of liver regeneration, inflammatory cytokines such as IL-6 and TNFα play critical roles [5]. Then, the proliferation phase promotes hepatocytes to re-enter the G1 phase with the stimulation of growth factors such as HGF and EGF [6]. In the terminal phase, TGFβ negatively regulates hepatocyte proliferation and participates in remodeling processes [7]. However, the depletion of any one of the above factors alone does not lead to incomplete or excessive liver regeneration, and the mechanism of liver regeneration regulation remains elusive. In addition, a balance between stimulatory and inhibitory factors of hepatocyte proliferation is necessary for normal liver regeneration. Liver regeneration is different from tumor proliferation, and proliferation inhibitors ensure the correct direction of regenerative processes by preventing proliferation that results in oncogenesis [8].

Transmembrane and ubiquitin-like domain-containing 1 (Tmub1), also known as hepatocyte odd protein shuttling (HOPs) or dendritic cell-derived ubiquitin-like protein (DULP), is upregulated in liver regeneration and was first reported by Della-Fazia et al. [9]. Tmub1 is upregulated in the regenerating liver after PHx and is actively exported from the nucleus in dividing cells but predominantly located in the nucleus during growth arrest [10]. Tmub1 is also an essential component of centrosome assembly during the cell cycle, suggesting the importance of Tmub1 in cell cycle regulation. In addition, Tmub1 also plays certain roles in the central nervous system. It regulates locomotor activity and wakefulness by interacting with calcium modulating ligand (CAMLG) [11] and facilitates the recycling of the AMPAR subunit

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GluR2 to the cell surface in the mouse brain [12]. In our previous studies, we found that Tmub1 is a negative regulator of rat hepatocyte proliferation [13]. Tmub1 is involved in the IL-6-induced proliferation pathway in the liver [14], C/EBPβ is a key transcriptional factor that regulates Tmub1 expression [15], and miR-27a/b is essential for the posttranscriptional modification of Tmub1 expression during liver regeneration [16]. However, the physiological functions of Tmub1 remain largely unknown, especially the in vivo effect of Tmub1.

In this study, we found that Tmub1 impairs liver regeneration by negatively regulating hepatocyte proliferation via STAT3 signaling. The role of Tmub1 in liver regeneration provides a potential strategy for the management of liver regeneration.

2. Materials and methods

2.1. Mice

Nine- to 10-week-old male C57BL/6 mice weighing 23 to 25 g were used in this study. Mice were obtained from the experimental animal center at the Third Affiliated Hospital, Third Military Medical University. All animals were maintained in the same experimental environment with a 12 h light/dark cycle, and food and water were provided ad libitum. All animal experimental procedures were carried out in accordance with the regulations of the Animal Care and Use Committee of the Third Military Medical University, China. All animal protocols were approved by the Medical Ethics Committee of the Third Military Medical University.

2.2. Partial hepatectomy

Classical 70% hepatectomy was used to initiate mouse liver regeneration. In brief, mice were anesthetized by inhalation of isoflurane (2%), and then the left lateral lobe and the median lobe plus the gall bladder were removed by ligation at their stem and excision, as described previously [17]. Sham-operated mice solely underwent laparotomy and liver manipulation. The animals were sacrificed at the indicated time points (0 and 72 h) after the surgery. Blood samples were drawn and centrifuged. Liver tissues were harvested and fixed with 4% paraformaldehyde for histological examination or snap-frozen in liquid nitrogen and stored at −80 °C for molecular and biochemical analyses.

2.3. In vivo transfection

In vivo Tmub1 overexpression in mice was achieved with plasmid DNA using Entranster in vivo transfection reagent (Engreen Biosystem Co.) via tail vein injection at the indicated time points (48 h before partial hepatectomy and 24 h after the surgery). The transfection was performed following the manufacturer's instructions. An empty vector was used as a negative control. The mouse Tmub1 overexpression plasmid was purchased from Cyagen Biosciences.

2.4. Cell culture and transfection

The human normal hepatocyte cell line Lo2 was purchased from the cell bank of Academia Sinica (Shanghai, China). The cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C with 5% CO_2. Human Tmub1 overexpression and shRNA plasmids were purchased from Cyagen Biosciences. The shRNA sequence was 5′-GACACCATTG GCTCCTTGAAA-3′. Plasmid DNA or shRNA was transfected using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's instructions.

2.5. 5-Ethynyl-20-deoxyuridine (EdU) assays

Lo2 cells were seeded onto 24-well plates. Twenty-four hours later, the cells were transfected with Tmub1, shTmub1 or NC vectors. Thirty-six hours after transfection, the cells were incubated with stattic (MCE) or oncostatin M (OSM, purchased from Sino Biological Inc.) for an additional 24 h; DMSO was used as a negative control. Cell proliferation was then determined in vitro with an EdU DNA Proliferation In Vitro Detection kit (KeyGen Biotech) based on the manufacturer's instructions.

2.6. Western blotting

Cell lysates were prepared with RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail (CoWin Biosciences). Nuclear and cytoplasmic extracts were separated according to manufacturer's instructions of Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). Protein concentrations were quantified with a BCA protein assay kit (CoWin Biosciences) according to the manufacturer's protocol. Protein extracts (20 μg) were denatured in Laemmli buffer and then loaded and separated by gel electrophoresis on a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and blocked with 5% nonfat dry milk buffer for 1 h. The membranes were incubated with the indicated primary antibodies at 4 °C overnight and with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Anti-STAT3, anti-phospho-STAT3 (Tyr705), anti-Tmub1, anti-cyclin D1, anti-Histone H3, and anti-β-tubulin antibodies were purchased from Abcam. Anti-STAT1 and anti-phospho-STAT1 (Y701) antibodies were purchased from abclonal. Anti-flag, anti-MCM2, anti-β-actin, HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Proteintech. An anti-c-myc antibody was purchased from Wanleibio.

2.7. Histological analysis

Livers were harvested and fixed in 4% paraformaldehyde for at least 24 h. Then, liver samples embedded in paraffin were cut into 5 μm-thick sections. Then, the liver tissue sections were stained with hematoxylin-eosin (H&E) using standard procedures. For immunohistochemistry, sections were rehydrated and processed for antigen unmasking and were then incubated with primary antibodies at 4 °C overnight followed by incubation with peroxidase-conjugated secondary antibodies. All sections were counterstained with hematoxylin. Images were then obtained with a light microscope. An anti-phospho-H3 (S10) antibody was purchased from Abcam, and an anti-Ki-67 antibody was purchased from Wanleibio.

2.8. Immunofluorescence

Lo2 cells were seeded onto glass slides in 24-well plates. Twenty-four hours later, the cells were transfected with Tmub1 or NC vectors. Forty-eight hours later, the glass slides were removed, and the cells were fixed with 4% paraformaldehyde at room temperature for 15 min. The cells were blocked with 5% goat serum and 0.5% Triton X-100 in PBS and then incubated with anti-STAT3 or anti-phospho-STAT3 (Tyr705) primary antibody (Abcam) overnight at 4 °C. After incubation with an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (Proteintech), Hoechst 33324 was used for nuclei staining. Images were then obtained with a fluorescence microscope.

2.9. Co-immunoprecipitation

Cells were collected and lysed with 500 μl of cell lysis buffer for Western blotting and IP (Beyotime Biotechnology) containing protease inhibitor cocktail (1:50) and phosphatase inhibitor cocktail (1:50). Next, 60 μl of protein G magnetic beads (Bio-Rad) was incubated with 4 μg of anti-flag (Sigma-Aldrich), anti-phospho-STAT3 (Tyr705) antibody (Abcam) or anti-STAT1 antibody (abclonal) respectively on a rotating platform for 30 min at room temperature and was then incubated

with 500 μl (1 mg) of cell lysate and rotated overnight at 4 °C. Normal rabbit IgG was used as a negative control. The IP products were eluted with 40 μl of 1× Laemmli buffer and incubated for 10 min at 95 °C. Western blot analysis was used for subsequent protein detection.

2.10. Chromatin immunoprecipitation (ChIP) assay

Lo2 cells were treated with 15 ng/ml human recombinant IL-6 (Novoprotein) or sterile water for 24 h, and then ChIP assays were performed with a ChIP kit (Cell Signaling Technology #9005) according to the manufacturer's instructions. Briefly, the cells were fixed with 1% formaldehyde to covalently crosslink the proteins to DNA, after which the chromatin was harvested. The crosslinked DNA was then digested and immunoprecipitated at 4 °C overnight with an anti-phospho-STAT3 (Tyr705) antibody (Abcam). Normal rabbit IgG was used as a negative control, and anti-histone H3 (Cell Signaling Technology) was used as a positive control antibody. The protein/DNA complexes were reverse cross-linked, and the DNA was purified using spin columns. Then, real-time quantitative PCR was performed to measure the enrichment of DNA fragments of the putative STAT3-binding sites within the Tmub1 promoter. The sequences of the PCR primers used for the Tmub1 promoter are as follows: forward: 5'-TGG GTGGAGGTGAGCGAATAGC-3′ and reverse: 5'-CATAGACCTTGGAACC TGGCAGAA-3′.

2.11. Luciferase reporter gene assay

For reporter gene assays, human Tmub1 promoter was cloned upstream of a firefly luciferase reporter gene (pGL3-basic, Promega). A persistently active mutant construct of STAT3, STAT3C (Stat3-C Flag pRc/CMV), was a gift from Jim Darnell (Addgene plasmid # 8722) [18]. Lo2 cells grown in 12-well plates were co-transfected with 0.8 μg/well pGL3/Tmub1 promoter or pGL3-basic, and with 0.8 μg/well of STAT3C or the control plasmid. Cells were also transfected with pRlc vector (Promega) for normalization. The luciferase activity was determined 48 h after transfection using a dual-luciferase reporter assay system (Promega).

2.12. Statistical analyses

The results are shown as the means ± S.E.M. The statistical analyses were performed using an unpaired Student's *t*-test, and $P < .05$ (two tailed) was considered statistically significant.

3. Results

3.1. Tmub1 inhibits mouse liver regeneration after PHx

To investigate the effects of Tmub1 on liver regeneration in vivo, Tmub1 overexpression plasmids or negative control plasmids were transfected into C57BL/6 mice, followed by 70% hepatectomy (Fig. 1A). Seventy-two hours after the surgery, we found that overexpression of Tmub1 significantly impaired liver mass reconstitution after calculating the liver/body weight ratios (Fig. 1B). Additionally, Tmub1 interfered with liver recovery, as revealed by the alanine transaminase (ALT) and aspartate transaminase (AST) serum levels (Fig. 1C). In addition, histological examinations also confirmed that Tmub1 impaired hepatocyte recovery, as more degenerated hepatocytes (indicated by abnormal retention of lipids within these hepatocytes) were visible in the Tmub1 overexpression group than in the control group after HE staining (Fig. 1E). Furthermore, Ki-67 immunohistochemistry showed that the proliferation levels in Tmub1-overexpressing mice were significantly lower than those in the control mice. Phospho-histone H3, a mitotic marker specifically expressed during the G2/M phase, showed a corresponding decrease in the Tmub1-overexpressing mice (Fig. 1F). Western blotting assays showed that Tmub1 overexpression were achieved by the in vivo transfection (the upper 27 kDa band and the lower 24 kDa band represents the two isoforms of Tmub1), and that 72 h after hepatectomy, the expression of the proliferation marker MCM2 and the cell cycle regulator cyclin D1 and the phosphorylation level of the transcription factor STAT3 were significantly reduced in the Tmub1-overexpressing mice (Fig. 1D). Taken together, our findings suggest that Tmub1 inhibits mouse liver regeneration in vivo.

3.2. Tmub1 inhibits the phosphorylation and activation of STAT3

IL-6 is the major cytokine that activates STAT3 in hepatocytes and is consequently responsible for hepatocyte proliferation following partial hepatectomy. Once the transcription factor STAT3 is activated, it translocates into the nucleus and induces the transcription of appropriate IL-6-responsive genes. Among these, cyclin D1 and c-myc are two major downstream genes of STAT3 and are responsible for cell cycle progression [19]. To investigate the relationship between Tmub1 and STAT3, Tmub1 overexpression, Tmub1 knockdown and control expression were induced in the human normal hepatocyte cell line Lo2 by plasmid transfection. Western blotting showed that Tmub1 overexpression significantly decreased the phosphorylation of STAT3 and significantly reduced the expression of cyclin D1 and c-myc. Conversely, knockdown of Tmub1 significantly increased the STAT3 phosphorylation level and significantly elevated the levels of cyclin D1 and c-myc, while total STAT3 expression was not influenced by Tmub1 overexpression or knockdown (Fig. 2A). To further investigate whether Tmub1 regulates the subcellular localization of STAT3, the cytoplasmic and nuclear fractions were prepared from Lo2 cells and mouse livers 72 h after PHx, and the western blot analysis showed no changes in STAT3 and p-STAT3 intracellular distribution after Tmub1 overexpression. (Fig. 2B and C). In addition, immunofluorescence assays also showed no changes in STAT3 intracellular distribution after ectopic expression of Tmub1 (Fig. 2D). These data revealed that Tmub1 inhibits the phosphorylation and activation of STAT3 but does not affect the expression or the subcellular localization of STAT3.

3.3. Tmub1 inhibits hepatocyte proliferation via targeting STAT3 signaling

Upon use of the STAT3 inhibitor stattic, the phosphorylation levels of STAT3 were significantly reduced in Lo2 cells (Fig. 3A). The STAT3 activator OSM significantly increased STAT3 phosphorylation (Fig. 3B). An EdU cell proliferation assay showed that Lo2 cell proliferation was significantly reduced by Tmub1 overexpression and was rescued by an additional 24 h of treatment with 2 ng/ml OSM (Fig. 4A). In contrast, 24 h of treatment with 5 μM stattic abolished the promotive effect of Tmub1 knockdown on proliferation in Lo2 cells (Fig. 4C). To further confirm the activation state of STAT3 in Lo2 cells ectopically expressing Tmub1, Western blotting assays were performed. As expected, p-STAT3, cyclin D1 and c-myc were significantly reduced in Tmub1-overexpressing cells, while additional administration of OSM abolished the effect of Tmub1 overexpression (Fig. 4B). In contrast, knockdown of Tmub1 significantly promoted the expression of p-STAT3, cyclin D1 and c-myc, while stattic counteracted this effect (Fig. 4D). In summary, these data indicate that Tmub1 inhibits hepatocyte proliferation by at least partially inhibiting the phosphorylation and activation of STAT3.

3.4. Tmub1 inhibits STAT3 signaling in a negative feedback loop

In our previous study, we found that IL-6 may enhance the expression of Tmub1 through the transcription factor C/EBPβ during rat hepatocyte proliferation. Moreover, STAT3 and AP-1 binding sites were found within the promoter region of rat Tmub1 [15]. Since STAT3 is the most characterized downstream transcription factor of IL-6, we studied the possible binding of STAT3 to the Tmub1 promoter in human hepatocytes. Based on bioinformatics analysis, a fragment sequence that

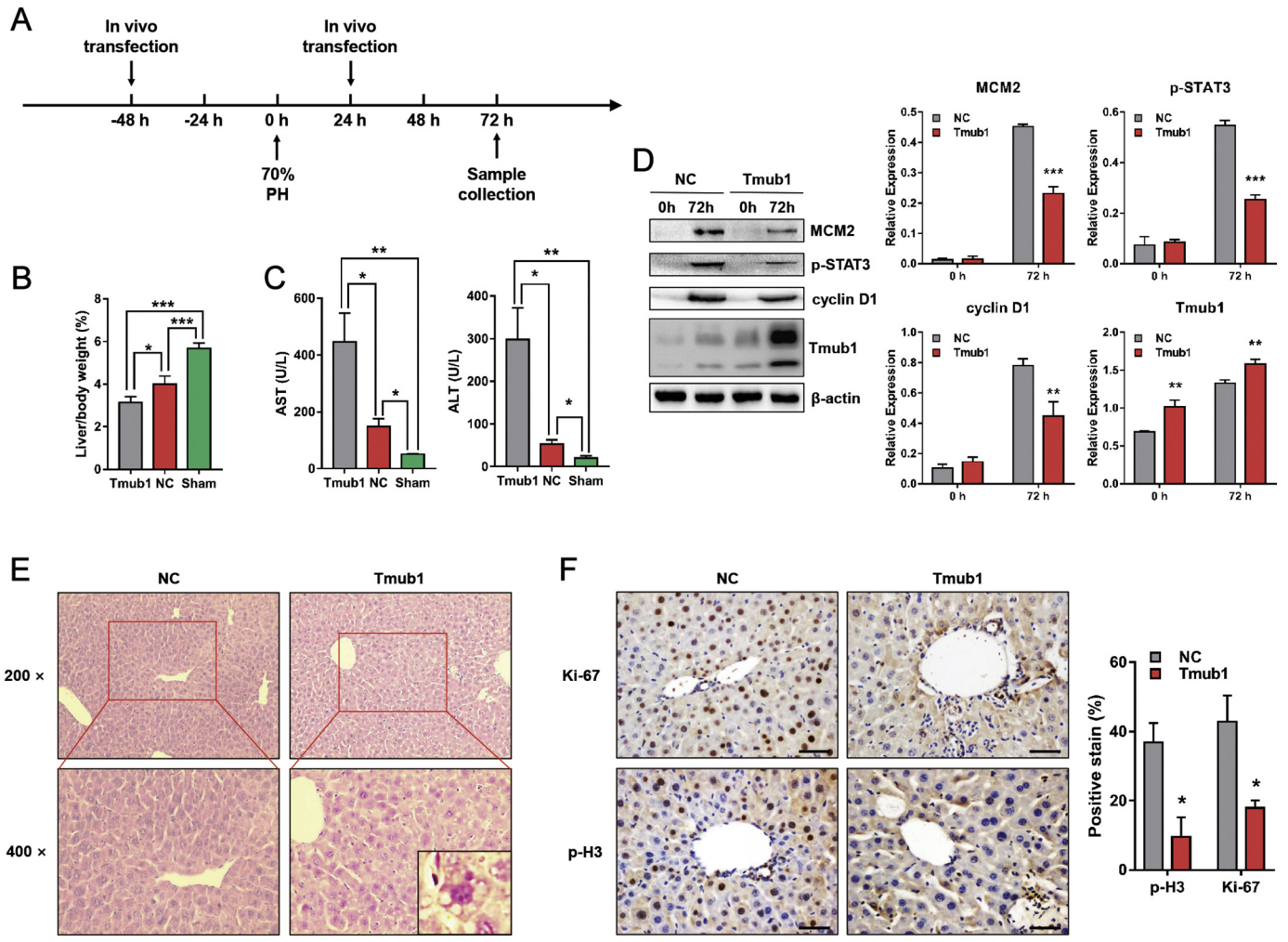

Fig. 1. Tmub1 inhibits mouse liver regeneration after PHx.
(A) Schematic of the experimental protocol for in vivo transfection and PHx. (B) Liver-to-body-weight ratio in mice transfected in vivo with Tmub1-overexpression and negative control plasmids at 72 h after PHx. The sham operated mice were used as the baseline control. (C) Serum AST and ALT levels 72 h after PHx. (D) Western blotting analysis and densitometric analysis of Tmub1 and proliferation-related proteins from liver extract at 0 h and 72 h after PHx. The levels of the indicated proteins are expressed relative to the levels of β-actin. (E) Hematoxylin-eosin staining and (F) immunohistochemical staining of Ki-67 and p-histone H3 (S10) in liver sections from Tmub1-overexpressing and NC livers at 72 h after PHx (400× magnification). Insert: representative image of degenerated hepatocytes. All data are shown as the means ± SEM; $n = 3$; $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ compared with NC mice.

contained one STAT3 binding site (−543 to −533 bp) within the human Tmub1 promoter region was selected and used as the target sequence in the following experiment (Fig. 5A). ChIP assays were then conducted to determine whether STAT3 could bind to the Tmub1 promoter and regulate its expression in human liver Lo2 cells. The PCR results demonstrated that the DNA sequence binding to STAT3 contained the promoter sequence of the Tmub1 gene (Fig. 5B). In addition, treatment with IL-6 significantly increased the binding between STAT3 and the Tmub1 promoter (Fig. 5C). Using the constitutive active STAT3 construct STAT3C, we found that active STAT3 promoted Tmub1 expression (Fig. 5D). Further luciferase reporter gene assays confirmed that activated STAT3 significantly increased Tmub1 promoter activity (Fig. 5E).

STAT1 and STAT3 have opposing roles in liver regeneration. To preliminarily investigate the possible interactions between Tmub1 and STAT1/3, co-IP assays were performed in flag-Tmub1-transfected Lo2 cells (since we did not find any Tmub1 antibodies suitable for IP assays), and the results revealed that Tmub1 and STAT3 may form a protein complex (Fig. 5F, upper). Further co-IP assays in normal Lo2 cells confirmed this finding (Fig. 5F, lower). However, the interaction between Tmub1 and STAT1 were not observed in our study (Fig. 5H). We next examined whether Tmub1 affects STAT1 expression by western blot assays, and the results showed that Tmub1 up-regulated the expression of STAT1 (Fig. 5G). These results indicated that Tmub1 may regulate the role of different STAT isoforms though different mechanisms.

The above results indicated that upon IL-6 stimulation, phosphorylated and activated STAT3 may directly bind to the Tmub1 promoter to enhance its expression, and this increased Tmub1 may in turn to form a protein complex with phosphorylated STAT3 and inhibit the function of STAT3 (Fig. 6). This Tmub1-STAT3 negative feedback loop may be an efficient self-regulatory mechanism in liver regeneration.

4. Discussion

Tmub1 is mainly regarded as a cell cycle-associated protein in the process of liver regeneration. After partial hepatectomy, Tmub1 is up-regulated in the regenerating liver [9]. Additionally, Tmub1 is over-expressed in hepatoma cells and plays a negative regulatory role in the process of hepatocyte proliferation [10]. Our previous study on rat hepatocytes showed that overexpression of Tmub1 significantly inhibits hepatocyte proliferation by decreasing the expression of cell cycle-

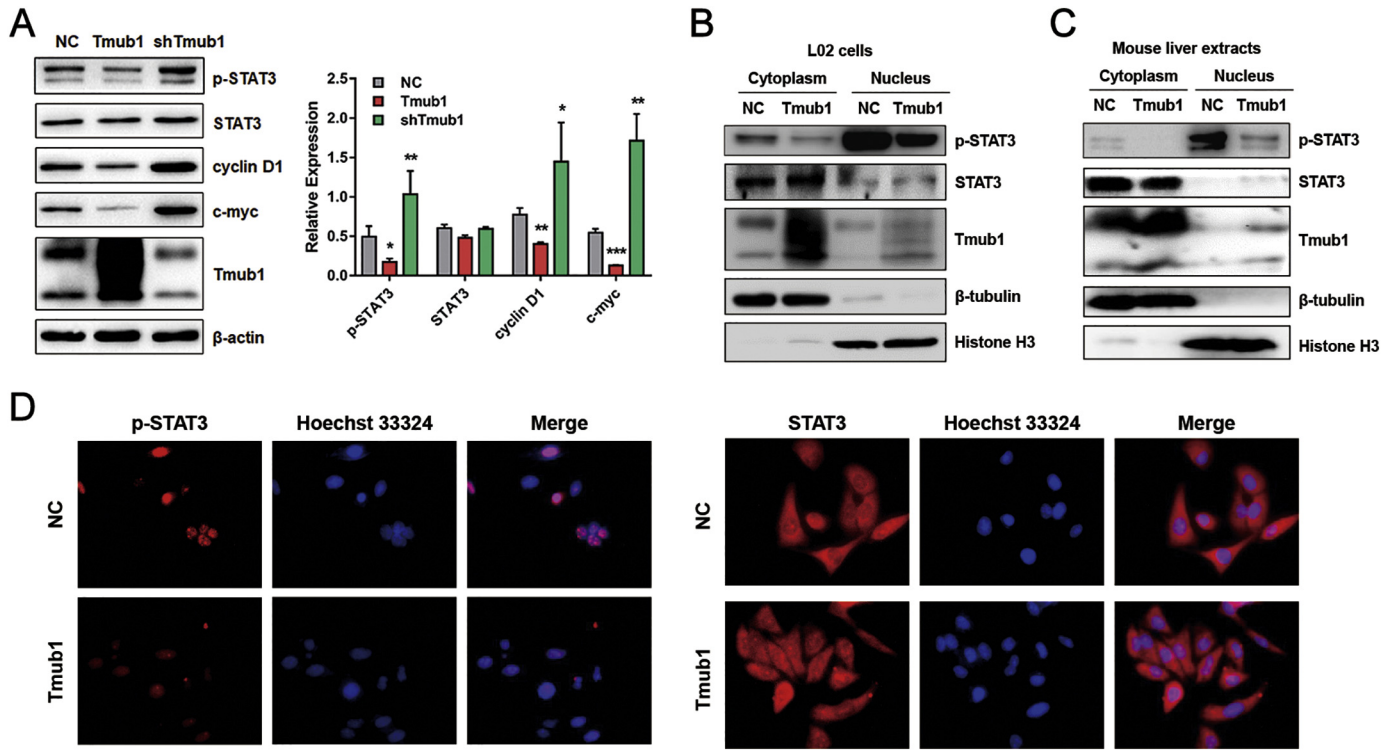

Fig. 2. Tmub1 inhibits the phosphorylation of STAT3 but not the localization of STAT3.
(A) Western blotting analysis of Tmub1 and STAT3 signaling proteins in Tmub1-overexpressing (Tmub1), Tmub1-knockdown (shTmub1) and negative control (NC) Lo2 cells. Densitometric analysis of the immunoblots was conducted relative to β-actin. All data are shown as the means ± SEM; n = 3; *p < .05; **p < .01; ***p < .001 compared with the NC group. (B) Western blotting analysis of cytoplasmic and nuclear STAT3 in Tmub1 and NC Lo2 cells and (C) mouse liver extracts 72 h after PHx. β-tubulin and Histone H3 were used as loading controls. (D) Immunofluorescence for STAT3 and p-STAT3 in Tmub1 and NC Lo2 cells. Hoechst 33324 was used for nuclear staining (200× magnification).

related genes. In contrast, Tmub1 knockdown significantly promotes hepatocyte proliferation [13]. However, whether Tmub1 ectopic expression alters the process of liver regeneration in vivo has not yet been determined. Here, we report for the first time that the upregulation of Tmub1 significantly harmed the regeneration of mouse liver, supporting the findings of in vitro studies on hepatocytes.

Liver regeneration is controlled by a wide variety of cytokines, growth factors, and hormones and their downstream signaling pathways. Among the regulatory factors, IL-6 and its downstream molecule STAT3 have been extensively studied [20,21]. As one of the triggers of liver regeneration, IL-6 is immediately produced by Kupffer cells after PHx. Upon IL-6 stimulation, STAT3 rapidly becomes phosphorylated and translocates into the nucleus to induce target gene expression [22]. The target genes include cyclin D and c-myc, which induce the G1-S phase transition in the cell cycle, as well as bcl-2, bcl-xl, mcl-1, and c-Flip, which protect against hepatocyte apoptosis [23]. However, although most investigators hold that IL-6 plays an important initiating and promoting role in liver regeneration [24,25], there is still some controversy regarding the role of IL-6 in liver regeneration. Blindenbacher et al. reported that IL-6 KO mice had higher mortality after PHx,

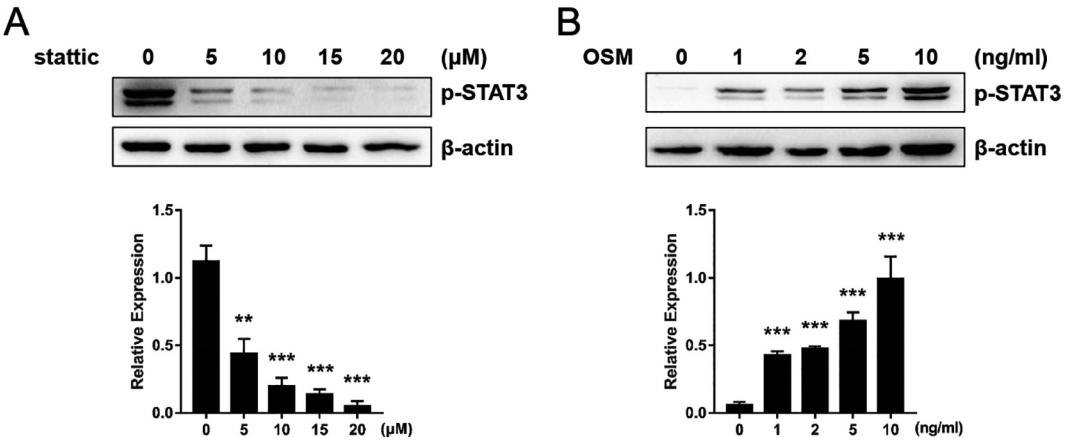

Fig. 3. Dose-dependent effect of the STAT3 activator and inhibitor.
(A) Western blotting and densitometric analysis of p-STAT3 in Lo2 cells after 24 h of treatment with the indicated concentrations of the STAT3 inhibitor stattic. (B) Western blotting and densitometric analysis of p-STAT3 in Lo2 cells after 24 h of treatment with the indicated concentrations of the STAT3 activator OSM. All protein expression was calculated relative to that of β-actin. All data are shown as the means ± SEM; *n* = 3; **p < .01; ***p < .001 compared with the NC group.

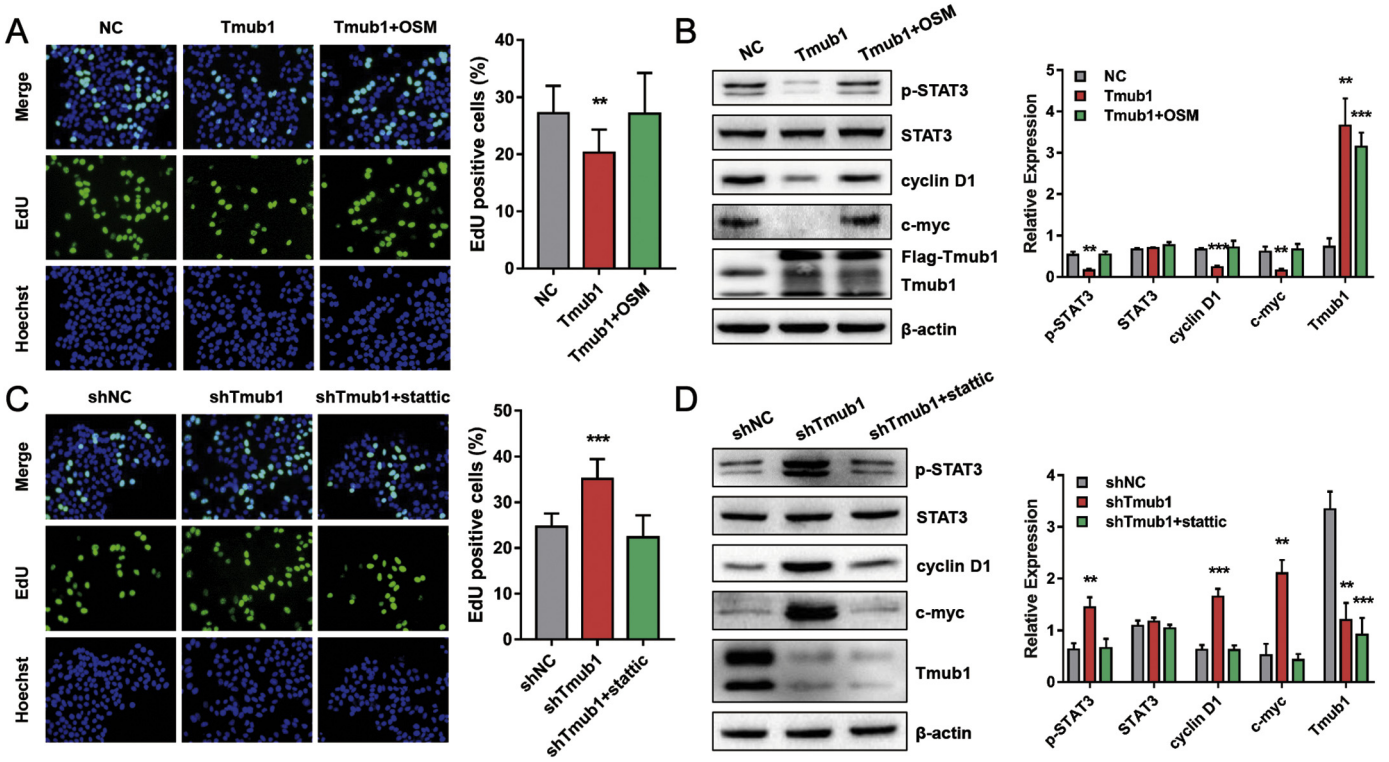

Fig. 4. Tmub1 inhibits hepatocyte proliferation via STAT3 signaling.
(A) An EdU cell proliferation assay demonstrated that the STAT3 activator OSM reversed the suppressive effect of Tmub1 on the proliferation of Lo2 cells. Nuclei were counterstained with Hoechst 33324 (200× magnification). (B) Western blotting analysis and densitometric analysis of Tmub1 and STAT3 signaling proteins in the above Lo2 cells. (C) An EdU cell proliferation assay demonstrated that the STAT3 inhibitor stattic reversed the promotive effect of shTmub1 on the proliferation of Lo2 cells. Nuclei were counterstained with Hoechst 33324 (200× magnification). (D) Western blotting analysis and densitometric analysis of the indicated proteins. All protein levels were calculated relative to those of β-actin. All data are shown as the means ± SEM; n = 3; **p < .01; ***p < .001 compared with the NC group.

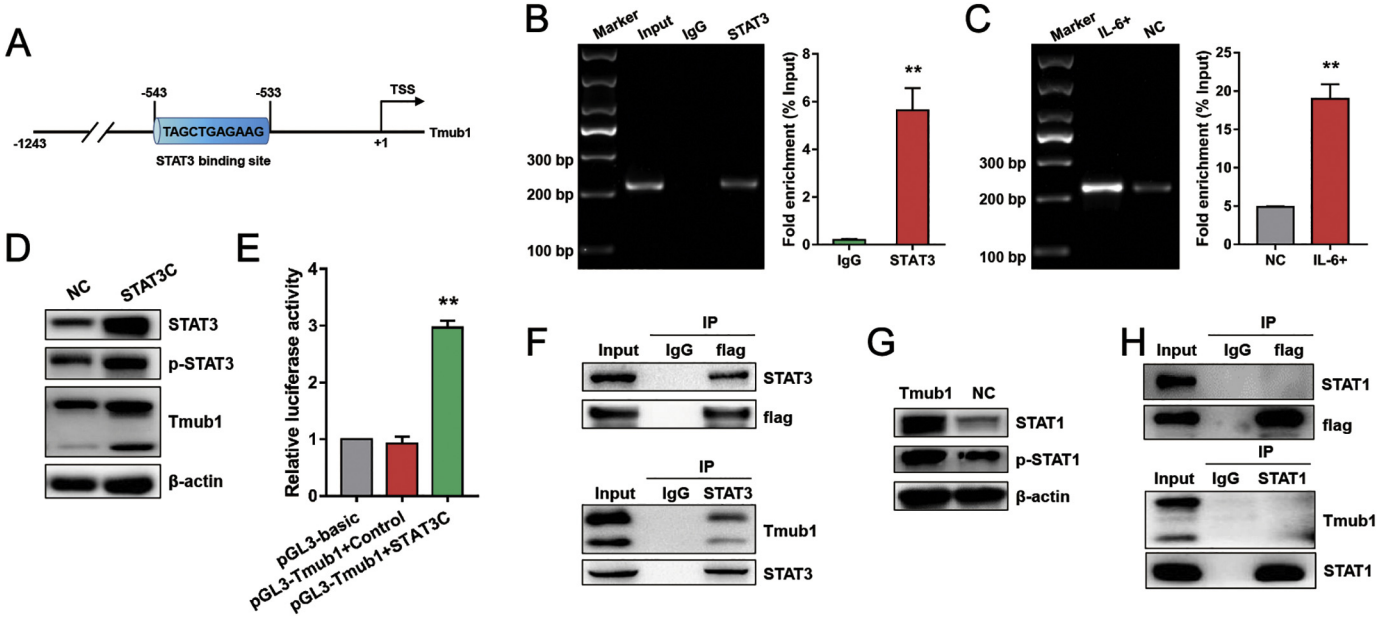

Fig. 5. Interactions between Tmub1 and STAT3.
(A) Diagram of the Tmub1 promoter and the putative STAT3 binding site. (B) ChIP-qPCR assays revealed that STAT3 binds the Tmub1 promoter. IgG was used as a negative control. (C) ChIP-qPCR of STAT3 and the Tmub1 promoter in IL-6-treated (IL-6 +) and untreated Lo2 cells (NC). (D) Western blotting analysis of Tmub1 in Lo2 cells after 48 h of transfected with the constitutive active STAT3 mutant STAT3C. (E) Luciferase reporter gene assay in Lo2 cells with or without STAT3C transfection. Luciferase activity was compared with that of pGL3-basic transfected group. (F) Upper: co-IP assays of Tmub1 and p-STAT3 in flag-Tmub1-transfected Lo2 cells. Lower: coimmunoprecipitation of p-STAT3 and Tmub1 in normal Lo2 cells. (G) Western blotting analysis of STAT1 and p-STAT1 in Tmub1 or NC Lo2 cells. (H) Upper: co-IP assays of Tmub1 and STAT1 in flag-Tmub1-transfected Lo2 cells. Lower: coimmunoprecipitation of STAT1 and Tmub1 in normal Lo2 cells. The data are shown as the means ± SEM; n = 3; **p < .01 compared with control.

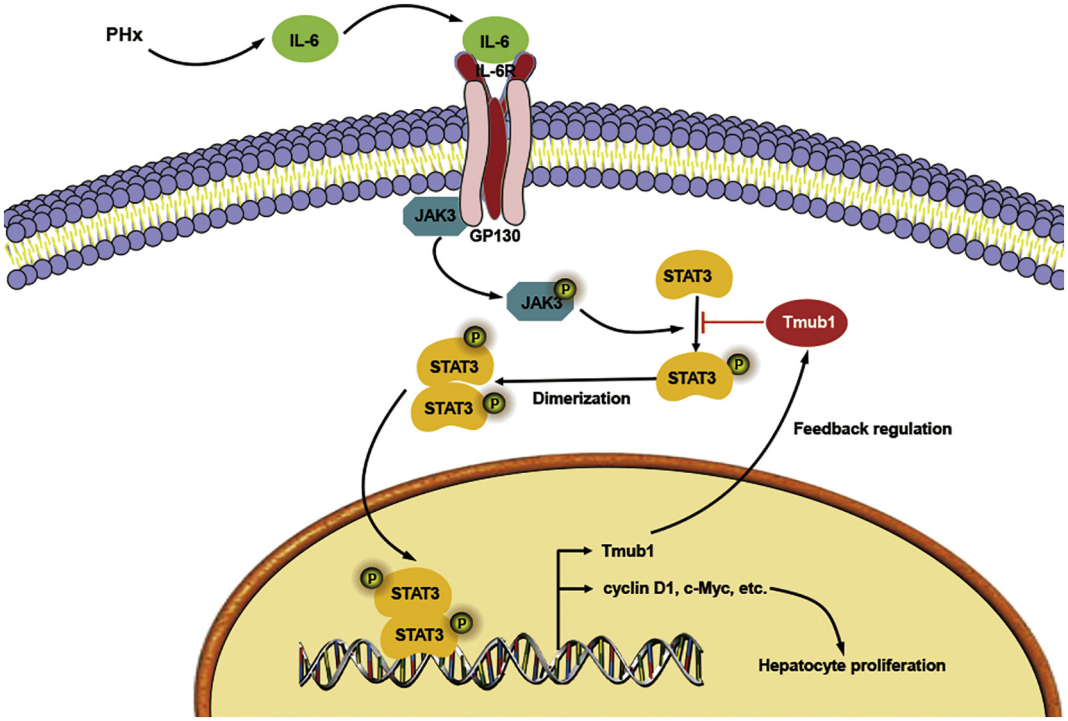

Fig. 6. Schematic diagram of the Tmub1 and STAT3 negative feedback loop in proliferating hepatocytes.

but the surviving IL-6 KO mice had similar hepatocyte proliferation to that of wild-type mice [26], and Sun et al. reported that IL-6 KO mice and WT mice had similar hepatocyte proliferation rates after PHx [27]. The reasons for these conflicting findings have yet to be illustrated. Since liver regeneration is precisely spatiotemporally regulated, promoting and inhibiting molecules act together or in a particular order to control the normal regeneration process. For example, activation of STAT3 also induces the expression of SOCS3, which in turn terminates STAT3 signaling and negatively regulates liver regeneration [28].

Clarifying the regulatory mechanism of Tmub1 expression is important in understanding the association between a proliferative promoting factor and a proliferative inhibitory factor in the hepatocyte regeneration process and the mechanism of hepatocyte homeostasis. In our previous study, we reported that the expression of Tmub1 can be induced by the additional administration of IL-6 in rat hepatocytes [14], partly through the action of the transcription factor C/EBPβ [15]. Since STAT3 is the most studied downstream effector of IL-6, in this study, we concentrated on investigating the interaction between Tmub1 and STAT3. Here, we introduce a role for Tmub1 as a negative regulatory molecule in STAT3 signaling during liver regeneration. Loss- and gain-of-function experiments supported the impact of Tmub1 on STAT3 signaling, in which Tmub1 significantly decreases p-STAT3 levels and the expression of STAT3 downstream genes without interfering with STAT3 translocation. Moreover, the effect of Tmub1 overexpression on hepatocyte proliferation can be restored by the STAT3 activator OSM, and the inhibition of cell proliferation caused by Tmub1 knockdown can be reversed with the STAT3 inhibitor stattic. These results suggested that Tmub1 negatively regulates hepatocyte proliferation via STAT3 signaling. In addition, the results from co-IP, ChIP and luciferase reporter gene assays demonstrated that the Tmub1 forms a protein complex with p-STAT3 and that p-STAT3 binds to the promoter region of Tmub1 in proliferating hepatocytes, a process that may be enhanced by IL-6 stimulation. These data suggested the presence of a Tmub1-STAT3 negative feedback loop in liver hepatocytes. Nevertheless, the detailed mechanisms of Tmub1-mediated inhibition of STAT3 phosphorylation remain unclear. For example, whether Tmub1 directly binds to STAT3 protein and blocks its phosphorylation, whether Tmub1 regulates the expression of other STAT3 inhibitors such as SOCS3, and whether Tmub1 disturbed the functional balance between STAT1 and STAT3 during liver regeneration, should be addressed in further studies.

The ubiquitin-proteasome system (UPS) is an important ATP-dependent proteolytic system [29]. In addition to the canonical ubiquitination pathway, proteins can also be modified through attachment to ubiquitin-like proteins (UBLs), such as NEDD8, UCRP and SUMO, which have conserved ubiquitin-like sequences and have demonstrated crosstalk with ubiquitination [30,31]. Tmub1 shares 26% identity and 34% similarity with ubiquitin; however, there is still a lack of evidence regarding whether Tmub1 can serve as a UBL to conjugate protein substrates through enzymatic cascades similar to those for ubiquitin conjugation. It is believed that the way in which ubiquitin is linked in polyubiquitin chains decides the fate of the modified proteins. Sequence analysis showed that Tmub1 does not possess the highly conserved C-terminus Gly-Gly required for ubiquitin conjugation or the Lys48 required for the formation of polyubiquitin chains to target substrates for degradation. However, three conserved Lys residues within ubiquitin, Lys29, Lys48 and Lys63, were found in Tmub1. These positions may potentially serve as sites for conjugation [32]. The only report so far is that Tmub1 may mediate the ubiquitination and degradation of the HMG-CoA reductase HMGCR. In this process, Tmub1 bridges SPFH2 to a membrane-bound ubiquitin ligase gp78 in endoplasmic reticulum membranes [33]. In this study, we also examined whether Tmub1 interferes with STAT3 ubiquitination and degradation. However, the expression of the STAT3 protein remained the same both before and after Tmub1 transfection, and the ubiquitination of STAT3 was unaltered (data not shown), suggesting that STAT3 may not be the substrate of the Tmub1 ubiquitin-like modification process.

In addition to the ubiquitin-like domain, the Tmub1 protein also contains two hydrophobic transmembrane domains. Studies have shown that Tmub1 is actively exported from the nucleus of hepatocytes in dividing cells, indicating that Tmub1 is a nucleus-cytoplasm shuttling protein [9]. An isoform with intermediate molecular weight is cleaved from the membrane and released into the cytosol to act as the shuttling protein. This process is controlled by the regulated intramembrane

proteolysis (RIP) system through a signal peptide peptidase located in the N-terminus of Tmub1 [34]. Our previous study revealed that Tmub1 can interfere with the binding of CAML to Cyclophilin B through its TM1 hydrophobic transmembrane domain [35]. Nevertheless, the mechanism and signaling pathway through which Tmub1 exerts its effects on cell biology remain vague. Future studies will be necessary to determine the detailed shuttling pattern of Tmub1 in the cell cycle, the different functions of Tmub1 in different subcellular locations, and the interactions between Tmub1 and its binding partners in both the nucleus and cytoplasm.

In summary, our study demonstrates that Tmub1 acts as a negative regulator of liver regeneration and that this effect is exerted at least partially by the inhibition of the phosphorylation and activation of STAT3. Tmub1 and STAT3 may form a negative feedback loop in proliferating hepatocytes. Our findings enrich the understanding of hepatocyte homeostasis and support the use of Tmub1 as a potential therapeutic target for the management of liver regeneration and the treatment of liver failure.

Declarations of interest

None.

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Authors' contributions

All authors participated in study design, data interpretation and analysis, and manuscript review. HWF, RD and YDZ performed the experiences. JHX contributed to data analysis. HWF prepared all figures and wrote the main manuscript. MGL and PC designed the present study.

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